Tissue Engineering Approaches to Cell-Based Type 1 Diabetes Therapy

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Type 1 diabetes mellitus is an autoimmune disease resulting from the destruction of insulin-producing pancreatic β -cells. Cell-based therapies, involving the transplantation of functional β -cells into diabetic patients, have been explored as a potential long-term treatment for this condition; however, success is limited. A tissue engineering approach of culturing insulin-producing cells with extracellular matrix (ECM) molecules in three-dimensional (3D) constructs has the potential to enhance the efficacy of cell-based therapies for diabetes. When cultured in 3D environments, insulin-producing cells are often more viable and secrete more insulin than those in two dimensions. The addition of ECM molecules to the culture environments, depending on the specific type of molecule, can further enhance the viability and insulin secretion. This review addresses the different cell sources that can be utilized as β -cell replacements, the essential ECM molecules for the survival of these cells, and the 3D culture techniques that have been used to benefit cell function.

Introduction

DIABETES MELLITUS is a condition characterized by deregulation of glucose stimulated insulin secretion (GSIS) by pancreatic β -cells. This deregulation can arise from autoimmune destruction of β -cells early in life (Type 1) or from a lack of insulin secretion or insulin resistance later in life (Type 2). The global incidence rate of diabetes mellitus was estimated to be ~ 280 million in 2010 and is predicted to increase to 440 million by 2030,¹ with type 1 diabetes accounting for ~ 5 – 10% of all diabetes mellitus cases.² The current gold standard for treatment of diabetes mellitus is administration of exogenous insulin in response to elevated blood glucose levels. Although this treatment requires constant glucose monitoring, the same level of control as endogenous insulin secreted from β -cells cannot be achieved. As a result, the patient is vulnerable to complications arising from hyperglycemia, such as severe dehydration, nausea, vomiting, increased urination, or even ketoacidosis in the short term.^{3,4} Over time nerve and blood vessels can be damaged, leading to neuropathy and blood vessel degeneration that manifests in symptoms ranging from numbness in extremities to complete loss of function and blindness. Although administration of exogenous insulin along with diet and exercise is sufficient to manage diabetes mellitus, this treatment does not cure the disease and requires continual patient compliance and vigilance. There is, therefore, a great need to develop alternative and long-term solutions to treating diabetes mellitus.

Cell-based therapies have been proposed as an alternative to exogenous insulin therapy, whereby islets, the endocrine cell clusters within the pancreas that contain β -cells, are implanted into the patient as a means to restore normal pancreatic function. Type 1 diabetes is defined by a loss of β -cell mass, and as such would benefit most from cell replacement therapy. The most notable breakthrough in cell-based therapies came with the advent of the Edmonton protocol in 2000, which involves transplanting islets obtained from cadaveric donors in conjunction with an immunosuppressive regimen. This protocol reversed hyperglycemia for 1 year in all seven patients who underwent islet transplantation.⁵ However, 5 years following transplantation, only 10% of patients remained insulin independent, with an average length of insulin independence of 15 months.⁶ With recent advancements in clinical approaches, patient response has been remarkably improved with 50% of patients remaining insulin independent for 5 years and more than 70% of implants retaining C-peptide secretion for 8 years.⁷ Despite this promising result, islet transplantation remains hampered by a lack of available donor tissue, the number of islets needed per patient, the need for immunosuppressants, and eventual loss of β -cell function over time.

Tissue engineering has the potential to overcome many of the shortcomings of the Edmonton protocol leading to increased longevity of islet transplantations. The idea of tissue engineering for cell-based type 1 diabetes therapy is to combine cells, such as islets or more specifically β -cells, with biomaterials that provide mechanical support and a

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suitable extracellular environment to maintain cell survival and function *in vitro* and *in vivo*. Recent findings, which will be described in this review, point toward three-dimensional (3D) culture systems as a promising platform to improve the clinical outcome of islet transplantations and for directing differentiation of stem cells into β -cells. Therefore, a tissue engineering approach may be critical to identifying a long-term therapeutic solution for type 1 diabetes. Based on recent studies in the literature, three main components appear to be key for islet-based tissue engineering: (1) viable cells that can form into cell aggregates and release insulin in response to glucose; (2) extracellular matrix (ECM) molecules incorporated into a biomaterial to support cell survival, enhance differentiation, and improve the function of insulin-producing cells; and (3) a mechanically supportive biomaterial used as a scaffold or encapsulation system to deliver cells *in vivo*. This approach is illustrated in Figure 1. Within the context of pancreatic tissue engineering, this review highlights recent key findings within each of the aforementioned categories.

Cell Sources and Culture Techniques

Many different cell types have the ability to serve as a transplantable cell source for the treatment of type 1 diabetes as long as they meet the clinical requirement, which is the release of insulin in response to elevated blood glucose without any adverse effects. In the pancreas, β -cells are the insulin-producing cells found in the Islets of Langerhans. Given the short supply of islets from donors, an active area

of research is the production of insulin-producing cells derived from stem or progenitor cells.

Islets

The Islets of Langerhans are clusters of endocrine cells within the pancreas, typically ranging in size from 50 to 300 μm in humans.⁸ Islets are comprised of α , β , γ , δ , and ϵ cells, which contribute to digestion and glucose metabolism in different ways. The β -cell is the insulin-producing cell within islets and its loss of function leads to type 1 diabetes. Within human Islets of Langerhans, β -cells are the predominant cell type (range 32–77%; average 59%) and are located predominantly at the periphery of intra-islet blood vessels, though interspecies variation exists in the percentage and location of β -cells within islets.⁹ β -Cells function by releasing insulin in response to elevated blood glucose, whereby insulin interacts with many other tissue-specific cells (e.g., fat, muscle, and liver) to initiate their metabolism of blood glucose for energy. Islets isolated from cadaveric donors are the primary cell source approved for clinical use, but are limited due to donor availability. Islets from nonhumans have been investigated as an alternative. Porcine islets are the most common source, as human and porcine insulin vary by one amino acid.¹⁰ The number of preclinical trials with porcine islets in human patients is extremely limited though porcine islets have survived and functioned for up to 6 months in nonhuman primates with immunosuppression.¹¹ Several preclinical trials using macro- or microencapsulation of porcine islets demonstrated efficacy for several years without the need for immunosuppressants.¹²

FIG. 1. Schematic depicting different approaches being investigated for pancreatic tissue engineering, whereby insulin-producing cells are isolated or differentiated, placed in a culture platform, and transplanted in order to reduce glycemic levels. ECM, extracellular matrix. Color images available online at www.liebertpub.com/teb

Nonetheless, islet transplantation remains an imperfect treatment due to limited long-term viability *in vivo* and the current need for a regiment of immunosuppressive drugs.¹³

Stem cells

Stem cells are defined by their ability to self-renew and differentiate into multiple cell types falling into one of two categories: pluripotent (having the ability to become all cell types in the body) or multipotent (having a restricted differentiation capacity). Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are the most commonly investigated for pancreatic differentiation, owing to their pluripotency, though investigators are searching for pancreatic stem cells and other β -cell progenitor cell types.

Embryonic stem cells. ESCs are derived from the inner cell blastocyst and characterized by their ability to differentiate into cells from all three embryonic germ layers. The basic concept currently employed for producing insulin-positive

cells from ESCs is to mimic the environment surrounding islets during development by delivering growth factors at defined intervals. The reader is referred to several excellent reviews that describe the signaling pathways involved in pancreatic development.^{14–17} The first documented attempt to produce insulin⁺ cells from human ESCs was introduced by Lumelsky *et al.*¹⁸ This article followed a protocol established for neuronal differentiation to produce insulin-positive clusters, as the developmental pathways for the central nervous system and pancreas are similar. Further approaches have attempted to create cells that more closely resemble pancreatic β -cells rather than insulin-positive neural-like cells. The most successful approaches have employed a multitude of growth factors and pharmacological agents aimed at recapitulating the signaling cascades relevant to pancreatic differentiation *in vitro*.¹⁹ Overall, there has been success in obtaining insulin⁺ cells *in vitro* from ESCs, comprising up to 25% of the final population, but glucose responsiveness *in vitro* remains a challenge (Fig. 2a).^{20–27} However, ESCs differentiated into β -cell precursors—characterized by transcription factors that

FIG. 2. Representative culture techniques for insulin-producing cells and stem cell differentiation into β -cells. **(a)** Directed differentiation of human embryonic stem cells (hESCs) into β -like cells, with up to 25% expressing C-peptide, a maker for insulin, reproduced with permission from The Company of Biologists.²⁷ **(b)** Pancreatic precursors derived from hESCs differentiate into functional glucose responsive islet-like structures that revert hyperglycemia when implanted *in vivo* and allowed to mature, reproduced with permission from Nature Publishing group.²⁹ **(c)** Suspension culture of MIN6 cells creates islet-like structures that reverse hyperglycemia *in vivo*, while single cells do not, reproduced with permission from Elsevier.⁶⁰ **(d)** Islets cultured in collagen alginate microbeads express more insulin than those in suspension culture (*, $P < 0.05$), reproduced with permission from Elsevier.¹⁰² **(e)** Aggregated pancreatic precursor cells cultured in poly(ethylene glycol) hydrogels with collagen I and gamma secretase inhibitor become insulin-positive β -like cells, reproduced with permission from Mary Ann Liebert, Inc., publishers.¹¹⁶ **(f)** Islets cocultured with human umbilical vein endothelial cells on a poly(lactic acid-co-glycolic acid) scaffold become vascularized upon implantation and reverse hyperglycemia.⁹⁹ Color images available online at www.liebertpub.com/teb

are critical to β -cell development and maturation, PDX1 and NKX6.1—have shown promise *in vivo*. When implanted into immunodeficient mice, these precursor cells were shown to mature into functional β -cells and reverse hyperglycemia (Fig. 2b).^{28,29} This finding indicates that the *in vivo* milieu provides critical cues for β -cell maturation and function, which has not yet been replicated *in vitro*, be it vasculature, ECM contacts, or other factors. However, the clinical use of partially differentiated ESCs remains limited due to their ability to form cysts and teratomas after transplantation. Despite the potential of ESCs to produce a limitless supply of pancreatic β -cells, current differentiation protocols will likely need to achieve *in vitro* glucose responsiveness and terminal differentiation before they can be applied in a clinical setting.

Induced pluripotent stem cells. iPSCs are derived from terminally differentiated somatic cells that, through the induction of genes found in pluripotent cells and down-regulation of genes of the differentiated state, regain traits of ESCs.^{30,31} This process offers an autologous cell source thus mitigating the need for immunosuppressants and overcomes the ethical concerns associated with ESCs. A few studies have subjected iPSCs to directed differentiation protocols similar to those used with ESCs and successfully produced insulin⁺ cells at similar percentages to that obtained for ESCs.^{25,32–34} Additionally, pancreatic precursor cells derived from murine or rhesus monkey iPSCs were found to reverse diabetes in immunodeficient mice after a month-long maturation period *in vivo*.^{35,36} The safety of iPSCs, which typically are produced through adenoviral delivery systems that can activate known oncogenes, needs further investigation before clinical implementation can be pursued.³⁷

Mesenchymal stromal cells. Mesenchymal stromal cells (MSCs) are fibroblast-like cells derived in the bone marrow, which have the ability to differentiate down several cell lineages, notably osteogenic, chondrogenic, and adipogenic lineages.³⁸ Recent studies have explored the possibility of differentiating MSCs into pancreatic endocrine cells including β -cells, but with limited success.^{39–41} A few studies have injected MSCs into diabetic mice or pigs and demonstrated a reduction in blood glucose, though this appears to be due more to a regeneration of host β -cell mass rather than differentiation of MSCs into β -cells.^{42,43} β -Cell regeneration is believed to be due to the immunomodulatory and angiogenic effects of MSCs, though the exact mechanism is not known.⁴⁴ The most widely adopted use of MSCs is in coculture with implanted islets, which has been shown to improve the clinical outcome by revascularizing the transplant and protecting the islets from immune attack. The use of MSCs appears to be primarily immunomodulatory though the ability of these cells to differentiate into insulin-producing cells and is still being researched. A recent review summarizes the potential use of MSCs in islet transplantation.⁴⁵

Other pancreatic cell types. Attempts have been made to produce insulin⁺ cells from cells that have a developmentally similar background to β -cells. Pancreatic acinar and ductal cells have been proposed as a population of cells that can differentiate into β -cells.^{46–48} Other studies have investigated the feasibility of transdifferentiating hepatocytes or other endocrine cell types into β -cells, which share a

common progenitor with pancreatic β -cells.^{49–51} The basic concept involves dedifferentiating the cell population back to its progenitor state and then redifferentiating the progenitors into β -cells. To date, these cell populations and approaches have been unsuccessful at producing functional β -cells that reverse hyperglycemia *in vivo*.⁵² However, this is an active area of research and, if successful, would offer a viable cell source for tissue engineering. Pancreatic precursor cells, which can be readily isolated from prenatal mice, express a number of genes indicative of their commitment toward a pancreatic phenotype, but not insulin. As such, they may be a useful cell source to identify conditions to coax precursor cells into functional β -cells *in vitro*.

Cell lines for use in research

Although cell lines are not clinically relevant, owing to their origin from cancer cells, they can nonetheless serve as an important tool for studying β -like cells and developing strategies for pancreatic tissue engineering. Several cell lines retain some resemblance of normal β -cell function (weak glucose responsiveness and insulin secretion) and have been utilized in diabetes research.⁵³ From a tissue engineering perspective, some of the most commonly used pancreatic cell lines are RINm5F (rat), INS-1 (mouse), β -TC (mouse), and MIN6 (rat), with only MIN6 cells exhibiting glucose-stimulated insulin release.^{54–56}

Suspension

Suspension culture has been investigated as a means to culture and preserve islet structure *in vitro* prior to implantation or encapsulation in biomaterials. Long-term suspension culture has been largely unsuccessful due to the lack of cell–matrix contacts, which are lost during the isolation process leading to apoptosis.⁵⁷ On the other hand, suspension culture has been proposed as a method to aid in the differentiation of stem cells toward islet-like fates by creating and/or maintaining structures similar to aggregated endocrine cells.⁵⁸ For example, when preaggregated hESCs in the form of embryoid bodies were grown in suspension culture with differentiation factors, 5–20% of the total population was PDX1 positive.²³ There was large variability among aggregates, with some releasing C-peptide, a marker of insulin secretion, in response to glucose, while others were unresponsive to glucose stimulation, but none were able to lower blood glucose levels when implanted into a mouse diabetic model.²³ However in a different study, hESCs were aggregated into 100–200- μ m-diameter spherical clusters and subjected to a directed differentiation protocol in suspension culture.²⁹ The resulting cell population had 40–65% cells that showed immature endocrine or poly-hormonal markers, 16–47% cells that expressed pancreatic endoderm markers, and 7–32% that expressed PDX1⁺ cells. When implanted into diabetic mice, the aggregates were able to reverse diabetes after 9–11 weeks.²⁹ Overall, these promising studies demonstrate that it is possible to differentiate stem cells in suspension into pancreatic precursor cells, though direct comparisons to two-dimensional (2D) differentiations were not included.

Another benefit of suspension culture is scalability, as many commercial biological processes are performed in large perfusion bioreactors. The ability to create a clinically relevant number of β -like cells for transplantation under

good manufacturing practice and in a high-throughput manner will be essential for the clinical implementation of stem-cell-derived islet transplantation. In one study, a rat β -cell line (β -TC6) was grown in 3D rotational bioreactor containing collagen-coated microbeads acting as nucleation sites for cell aggregation and their subsequent release.⁵⁹ This culture method produced cells with higher gene expression levels of *PDX1*, *NeuroD*, *Insulin1*, and *Ngn3*—all β -cell or precursor markers—and were more responsive to glucose challenge when compared with 2D cultures. Another approach utilized a clinostat, a simulated microgravity generator, to create large numbers of MIN6 cell aggregates that were consistently $\sim 200\ \mu\text{m}$ and stained positive for C-peptide and insulin (Fig. 2c).⁶⁰ Combining defined differentiation approaches with high-throughput suspension culture systems will likely aid in producing a large number of β -like cells in a reproducible fashion.

Micropatterning/microwell technology

Micropatterning and microwell technologies have been utilized as a means to control aggregate size and study its effect on cell growth and differentiation of ESCs and the function of primary cell types. In microcontact printing, proteins are patterned onto a surface to localize cell attachment and promote cell aggregation. A study utilizing microcontact printing to create size-controlled hESC-derived aggregates demonstrated that smaller aggregates ($200\ \mu\text{m}$) expressed higher levels of endoderm markers while larger aggregates ($800\ \mu\text{m}$) expressed higher levels of neural markers.⁶¹ Similar microcontact printing techniques have been utilized to effectively differentiate hESCs into size-controlled islet-like clusters expressing PDX1, though the effect of aggregate size on differentiation efficiency was not considered.^{24,62}

Microwell technology is another technique to create size-controlled aggregates of defined dimensions created by micromolding or photocontact lithography. In one study, MIN6 cells were aggregated into clusters ranging from 100 to $300\ \mu\text{m}$ in diameter using microwells of defined sizes.⁶³ The aggregates released more insulin than an equivalent number of single cells, but no aggregate-size-dependent response was observed. Additionally, when these aggregates were encapsulated in a synthetic hydrogel, they remained

more than 90% viable after 1 week in culture while the viability of encapsulated single cells was below 10%. In another study, human pancreatic ductal epithelial cells cultured in a microwell encapsulation system (consisting of wells from 150 to $500\ \mu\text{m}$ in size with polycaprolactone-gelatin nanofibers lining the bottom of the wells) produced insulin after 21 days of culture.⁶⁴ Microwell and micro-patterned techniques offer platforms to produce well-defined aggregates; however, the optimal aggregate size for β -cell function and differentiation of stem cells into functioning β cells remains to be elucidated.

ECM Cues

Pancreatic islets are highly vascularized endocrine cell clusters that are in intimate contact with the basement membrane of endothelial cells and other exocrine cells in the pancreas. From a tissue engineering standpoint, knowledge of the extracellular signals present in pancreatic islets can be utilized to design biomimetic scaffolds capable of maintaining islet function. The ECM has several functions, such as providing structural support, acting as a reservoir for growth factors, and signaling to cells through integrin-mediated interactions.⁶⁵ Two recent reviews detail knowledge to date regarding essential ECM molecules and integrins that maintain islets *in vivo*.^{66,67} Laminin-111 has been implicated in the development of the pancreas, while laminins-332, 441, and 511 are found in the basement membrane of the adult pancreas.^{68,69} Collagens I, IV, and V are also components of the basement membrane, though other ECM molecules, such as vitronectin and fibronectin, are known to have roles in pancreatic development.^{70,71} A recent study of decellularized mouse pancreas identified several proteins present in the basement membrane, among them collagens I, III, V, and VI; laminins-111, 211, and 511; nidogens; and proteoglycans.⁷² However, the exact role of specific ECM molecules in maintaining islet function is still under investigation. Table 1 summarizes key findings for different ECM molecules and their effect on various insulin-producing cell types. While many of these studies utilized 2D cultures, they provide valuable information regarding the role of different ECM molecules in maintaining functional islets, which can be applied to the design of 3D biomaterials.

TABLE 1. SUMMARY OF EXPERIMENTAL FINDINGS INVOLVING INSULIN-PRODUCING CELLS CULTURED ON EXTRACELLULAR MATRIX

ECM component	Isoform	Cell types	Key findings
Laminin	111 332 411 511	Purified β -cells Adult rat islets ^{80,83,84} Fetal rat islets ^{75–77,81,82} hESCs ²⁴ MIN6 ⁶⁹	Enhanced viability ^{75–79} and insulin release ^{81–84} compared to TC plastic; less vimentin ⁺ mesenchymal cells ^{80,83,84} ; enhance differentiation of hESCs toward pancreatic cells ²⁴ ; enhanced insulin gene expression ⁶⁹
Collagen	I IV V	Rat islets ^{77,83} Human islets ^{81,82,88}	Facilitates attachment, spreading, and maintained viability of cultured islets ^{77,81,83} ; Epithelial-to-mesenchymal transition over time ⁸¹ ; increased insulin over islets on TC plates ^{82,88}
Fibronectin/vitronectin		Rat islets ^{77,78,82,83,90} hESCs ⁶²	Maintain viability and insulin release initially, though over time viability decreases ^{77,82,90} ; 3D morphology maintained in culture ^{78,83} ; aids in the differentiation of hESCs to pancreatic precursor cells ⁶²

ECM, extracellular matrix; hESCs, human embryonic stem cells; 3D, three-dimensional.

Laminin

Laminin is a major component of basement membranes, is present in blood vessels and acinar cells within the pancreas, is distributed within and around islets at the exocrine-endocrine transition, and is present in intra-islet blood vessels.^{73,74} Because it is an integral part of the extracellular environment of islets *in vivo*, many studies have investigated islet function on 2D cultures with different laminin isoforms. For example, laminin-111 associated with the pancreatic ductal epithelium has been shown to enhance differentiation and expansion of fetal mouse islets,⁷⁵ selectively enhance the number of insulin⁺ cells,⁷⁶ and maintain viability of adult rat islets for several days.⁷⁷ Human β -cells attached, spread, and survived on the basement membranes of lysed human 5637 cells, but not on lysed rat 804G cells, both of which are rich in laminins.⁷⁸ Rat islets, however, spread and secreted insulin with reduced apoptosis when cultured on the basement membranes of lysed 804G cells.⁷⁸ These studies suggest that cell-matrix interactions may be species specific.⁷⁹ In a separate study, human islets cultured on tissue culture polystyrene (TCPS) exhibited an epithelial-mesenchymal transition as evidenced by the expression of the mesenchymal marker vimentin, while those cultured on laminin isoforms (laminin-411 or 511) exhibited reduced proliferation but retained their β -cell phenotype and insulin gene expression levels.^{69,80} Fetal islets, dissociated and reaggregated into islet-like cell clusters and cultured on laminin-111, retained their aggregated structure, became less vimentin⁺ (<50% after 5 days), retained insulin gene expression, and had insulin release values 2.6 times higher than islets cultured on bovine serum albumin (BSA)-coated plates.^{81,82} Several studies have shown that islets cultured on laminin only weakly attached to the surface,^{83,84} suggesting that minimal spreading in addition to integrin-mediated events may be important to retaining insulin⁺ cells while minimizing the epithelial-mesenchymal transition. Additionally, laminin appears to enhance the differentiation of hESCs into insulin⁺ cells.²⁴ When hESCs were cultured on 100- μ m-diameter, microprinted laminin-coated coverslips under a directed differentiation protocol, the cell clusters exhibited gene expression profiles resembling definitive endoderm, the developmental precursor of pancreatic cells. When detached and further differentiated in suspension culture, a small fraction (<5%) of cells expressed pancreatic transcription factors PDX1 and NKX6.1.²⁴ Overall, it appears as though laminin plays an important role in maintaining islet function and insulin release *in vitro* and in supporting differentiation of hESCs into β -cell precursors.

Collagen

Collagen IV is also a major component of the basement membrane and collagen types I, IV, V, and VI are present at the exocrine-endocrine interface and in proximity to intra-islet endothelial cells.^{73,85} Collagens I, V, and VI are the most abundant isoforms within the islet-exocrine interface of the human pancreas, with only weak expression of collagen IV, though collagen IV is strongly expressed in the epithelial basement of the fetal pancreas.^{86,87} Collagen IV is present in the developing human pancreas and is implicated in the development of islet structure as evidenced by its proximity to budding clusters of insulin- and glucagon-positive cells.⁸⁷ In 2D culture, rat islets cultured on collagens I and IV re-

mained 60% and 89% viable, respectively, while over 90% of those cultured in suspension underwent apoptotic death after 48 h.⁷⁷ Within 24 h of plating, islets on collagens I and IV had four and six times the insulin release, respectively, of those on BSA-coated or standard poly-D-lysine plates.^{82,88} However, after 5 days of culture, 81% of islets cultured on collagen IV were vimentin⁺, with a 70% reduction in insulin transcription compared with day 0,⁸¹ indicating their transition into mesenchymal-like cells.⁸³ Interestingly, islets that form a monolayer on a collagen I surface followed by overlaying a second layer of collagen above the cells were able to form islet-like aggregates and maintain a basal level of insulin release over 8 weeks, implicating 3D matrix contacts as being important to preserving islet structure *in vitro*.⁸⁹ Despite the ability of islets to attach and proliferate on collagen I- and IV-coated surfaces, the observed epithelial-to-mesenchymal transition limits the usefulness of these substrates for *in vitro* 2D culture. As collagen IV is predominantly observed in the developing pancreas and is believed to facilitate the formation of islets, it may be a more suitable substrate for the directed differentiation of stem cells.⁸⁷ Other isoforms, like collagens V and VI, may be successful at enhancing islet function *in vitro*, though little work exists using these substrates.

Fibronectin/vitronectin

Fibronectin and vitronectin are present in the developing pancreas, but are not present in abundance in adult islets.^{67,87} Fibronectin is expressed in blood vessels and ductal cells of the developing pancreas while vitronectin is expressed in epithelial cells adjacent to ductal structures.⁸⁷ Despite the uncertain role of fibronectin and vitronectin in mature islets, both have been studied in 2D culture of islets. For example, rat islets cultured on fibronectin exhibited initially high viability (85–90%) after 24 h, which decreased to 43% after 7 days.^{77,90} Generally, islets do not attach strongly to fibronectin nor do they spread during culture.⁷⁸ Despite the weak binding to fibronectin or vitronectin, islets cultured on these substrates released 3.6 and 5.4 times more insulin, respectively, than those on BSA-coated plates after 24 h.⁸² Islets cultured on fibronectin were less spread than those on collagens I and IV and even laminin, and appeared most normal in terms of morphology and insulin release.⁸³ However, viability was not retained in long-term studies.⁸³ Given the role of these two proteins in development, hESCs were grown and differentiated on microprinted 150–200- μ m spots of fibronectin and vitronectin. These proteins aided in hESC differentiation toward definitive endoderm, the precursor to gut, thyroid, pancreas, and liver, and interestingly to a greater degree when compared to other ECM proteins (collagens I, III, IV, and V and laminin).⁶² The hESCs plated on a combination of fibronectin and vitronectin and differentiated toward definitive endoderm had a higher number of definitive endoderm-positive cells than those plated on matrigel (81.4% vs. 59.1%). In cells differentiating toward definitive endoderm, the integrins ITGA5, ITGAV, and ITGB4 were found to be upregulated, all of which bind fibronectin and vitronectin. Selecting for cells that were ITGA5⁺/ITGAV⁺ led to subsequent enhancements in the differentiation efficacy toward primitive gut tube, indicating development toward stomach, liver, or pancreas. Fibronectin and vitronectin are important components of the

fetal pancreas, and may be useful in the differentiation of stem cells to islets but their importance in maintaining mature islets appears to be less clear, as long-term viability on these substrates is a concern.

Nonproteinaceous matrix components

Transmembrane glycoproteins in islets are comprised of glycosaminoglycans (GAGs), including heparin sulfate (HS) and chondroitin sulfate (CS), which modulate cellular interaction with ECM and growth factors. Perlecan, also known as heparin sulfate proteoglycan 2, and CS are also present in the basement membranes of islets, though the role of these molecules in islet function is unclear. The reader is referred to a recent and thorough review describing the structure and function of many proteoglycans and their proposed roles in islet function.⁶⁶ Recent work has demonstrated that insulin-producing cells within mouse islets express high levels of transmembrane HS associated with syndecan-4.⁹¹ Syndecan-4 is involved in FGF-2 signaling, a pro-survival factor, and therefore may be important to β -cell survival.⁹² Other studies have shown that HS is essential for islet survival, with intracellular levels of HS in dissociated islets diminishing 67% after isolation, corresponding to a twofold increase in cell death.⁹³ These studies illustrate that in addition to proteinaceous matrix of the basement membrane surrounding islets, transmembrane glycoproteins are critical to maintain during the isolation

procedure for survival and function postisolation.⁹⁴ Further work is needed to elucidate the nature of GAGs and glycoproteins in cell–ECM interactions so that this knowledge can be applied to designing functional materials to sustain islet function.

Three-Dimensional Biomaterials

Three-dimensional culture systems present several advantages over traditional 2D culture. By maintaining cells in more physiologically relevant structures and preserving cell–cell and cell–matrix contacts, the *in vivo* environment can be more closely imitated. This consideration is particularly relevant when islets are the cell population in culture, owing to the numerous cell–cell contacts among endocrine cells and cell–matrix contacts among endocrine cells and the surrounding basement membrane. With islet transplantation, the isolation process often destroys the extracellular environment leaving islets prone to a loss of function over time after transplantation. Restoring ECM contacts and mechanical support of isolated islets may increase the long-term success of islet transplantation. There are numerous examples demonstrating that 3D cell culture is more physiologically relevant than monolayer culture, ranging from cancer research and stem cell differentiation, to the *ex vivo* culture of many cell types.⁹⁵ Therefore, 3D culture is likely to play an important role in the *in vitro* culture of pancreatic cells and their delivery *in vivo*. Table 2 summarizes key

TABLE 2. SUMMARY OF THREE-DIMENSIONAL CULTURE TECHNIQUES FOR PANCREATIC TISSUE ENGINEERING

Culture platform	Modifications	Cell types	Key findings
Suspension		MIN6 ⁶⁰ β -TC6 ⁵⁹ hESCs ^{23,29} Mouse fetal pancreatic cells ⁵⁸ MIN6 ⁶³ Human ductal epithelial cells ⁶⁴ hESCs ^{24,62}	Directed differentiation into β -like cells that can reverse hyperglycemia ^{29,58} ; suspension culture of MIN6 aggregates lowers blood glucose levels ⁶⁰
Microwell/ micropatterning			Consistent production of size-controlled (200–800 μ m) aggregates ^{24,62–64} ; increased insulin release compared with single cells but no size-dependent correlation ⁶³
Porous scaffold (PLLA, PLGA)	Extracellular matrix; RGD	Islets ^{96,97,99,105,106,125} RIN ⁹⁸ Coculture with HUVECs and fibroblasts ⁹⁹	Cells cultured on a scaffold have increased viability and insulin release compared with those cultured in 2D ^{96–99,105,106,125} ; the inclusion of ECM molecules within the scaffolds enhances insulin release ^{97,98} ; islets implanted <i>in vivo</i> in a scaffold become vascularized and lower blood glucose levels ^{99,125}
Hydrogel	Collagens I and IV; laminin; GLP-1; IL-1; peptide motifs for collagens I and IV and laminin	Rat islets ^{104,108,107,121,126} Human islets ^{122,104} MIN6 ^{102,104,110,111,117–120} Rat pancreatic precursors ^{114–116} Coculture with MSCs ^{122,123} Mouse ESCs ¹⁰⁹ RIN ¹⁰³	Cells cultured in a hydrogel have higher viability compared with 2D ^{102,104,107,110,111,119–122,126} ; the inclusion of ECM molecules within the scaffolds enhances insulin release ^{110,111,108} ; aggregation of cells prior to encapsulation facilitates survival and insulin release ^{120,114–116} ; MSCs enhance insulin release ^{122,123} ; differentiation of stem cells to β -like cells when cultured in hydrogels ^{109,114–116} ; islets in hydrogels can reverse hyperglycemia ^{102,107,121,126}

HUVECs, human umbilical vein endothelial cells; MSCs, mesenchymal stromal cells; 2D, two-dimensional.

findings associated with culturing insulin-producing cells or stem cells in three dimensions.

Three-dimensional porous scaffolds

Seeding cells onto prefabricated porous scaffolds has been investigated as a means to enhance viability and function of isolated islets *in vitro* and to improve the outcomes of islet transplantations. For example, rat islets cultured in a porous poly(glycolic acid) (PGA) scaffold were nearly twofold more viable and had fourfold greater insulin secretion when compared to those cultured on untreated TCPS in 2D after 15 days.⁹⁶ In a separate study, human islets were entrapped in a collagen gel containing fibronectin and collagen IV within the pores of a poly(lactic acid-co-glycolic acid) (PLGA) scaffold.⁹⁷ Interestingly, the islets within the PLGA scaffold maintained a glucose stimulation index similar to that of fresh islets and better than those cultured in a 3D collagen I gel, but in the absence of the scaffold, or those cultured in suspension. Further, β -cell-specific gene expression levels were also elevated (e.g., three- to fivefold greater than 3D culture in ECM alone) in islets cultured within the PLGA/ECM scaffold, suggesting that a 3D structural support provided by the pores was critical to maintaining islet function.⁹⁷ In a different study, cells from an insulin-producing cell line (RIN-m5F) were cultured on a scaffold comprised of woven PLGA with the pores filled with a collagen I microfiber mesh, which was subsequently coated with ECM molecules (laminin, fibronectin, vitronectin, or type IV collagen) or poly (L-lysine).⁹⁸ Insulin secretion in the 3D scaffolds was approximately twice that of cells maintained in 2D culture. These results demonstrate the importance of both cell-matrix contacts and the structural support provided by a scaffold in sustaining islet function *in vitro* and in enhancing islet function upon transplantation.

Another benefit of using scaffolding materials is the ability to coculture multiple cell types and create environments resembling aspects of native tissue structures with ECM interactions and proximity to vasculature. This was demonstrated in a study whereby mouse islets, human umbilical vein endothelial cells, and human foreskin fibroblast mesenchymal cells were cocultured on porous PLLA/PLGA scaffolds (Fig. 2f).⁹⁹ This triculture system improved islet viability (75% viable after 4 weeks *in vitro*) compared to 2D controls (no survival past 2 weeks). In addition, the inclusion of fibroblasts and endothelial cells in 2D or 3D led to increased expression of several pancreatic genes from encapsulated islets (INS, GCG, PDX1, NKX6.1, and Glut2), which were further upregulated in 3D. Islets in the triculture 3D system had ~50% greater insulin release than those cultured in 2D. Islet function is mediated by interactions with surrounding tissue and vasculature *in vivo*, and these studies suggest that coculture with endothelial cells benefits *in vitro* function as well.

Hydrogels for cell encapsulation

Hydrogels are highly water-swollen crosslinked polymers that can be formed from biocompatible natural or synthetic polymers in aqueous medium and under benign conditions enabling encapsulation of cells.¹⁰⁰ As such, they have been the most widely investigated materials for delivery of pancreatic cells for diabetes therapy. Alginate microcapsules have been one of the most commonly investigated hydrogel materials for pancreatic tissue engineering. Alginate is an unbranched anionic polysaccharide comprised of repeating units of 1,4-linked

α -L-guluronate and β -D-mannuronate, which is derived from the cell walls of brown algae. Alginate binds divalent cations, such as Ca^{2+} or Ba^{2+} , and assembles into an “egg box” like crosslinked networks under cytocompatible conditions.¹⁰¹ Alginate microcapsules have been explored as a means to encapsulate individual islets though creating long-lasting, uniform, and stable microcapsules remains an issue. Various attempts have been made to modify alginate microcapsules either with ECM molecules to facilitate cell adhesion or different stabilizing crosslinkers. For example, rat islets were encapsulated in alginate or an alginate/collagen mixture (Fig. 2d).¹⁰² The alginate/collagen-encapsulated spheroids maintained their viability after 1 week (75%) while alginate-encapsulated spheroids were only 35% viable, which was lower than 2D cultures (50%). In a separate study, RIN-m5f cells were encapsulated in barium-alginate or calcium-alginate with or without poly-L-lysine or poly-L-ornithine and ECM molecules.¹⁰³ Barium was more stable than calcium in maintaining crosslinking owing to the fact that Ba^{2+} can bind mannuronate in addition to guluronate. The presence of poly-L-lysine or poly-L-ornithine in the barium alginate microbeads increased cell proliferation, while the inclusion of collagen or laminin enhanced insulin release. To create even more stable alginate microbeads, alginate was crosslinked with poly(ethylene glycol) (PEG) and used to encapsulate MIN6 cells, rat islets, and human islets.¹⁰⁴ In this hydrogel system, proliferation of MIN6 cells was significantly greater than alginate-only microbeads after 7 days of culture. While rat and human islets were glucose responsive, their GSIS (the ratio of insulin released under high-glucose stimulation to the insulin released under low-glucose stimulation) and cell viability was similar in the PEG alginate and alginate-only microbeads after 1 week. Alginate microcapsules are capable of encapsulating individual islets in a stable and mechanically supportive environment, an important consideration for successful long-term transplantations.

Several other naturally derived hydrogels have been studied for the encapsulation of islets or their precursors. For example, a peptide amphiphile nanostructured gel-like structure was engineered to contain the cell adhesion binding domain RGD and MMP-2 cleavable sequences to impart degradation.^{105,106} When rat pancreatic islets were encapsulated in these hydrogels, they had higher GSIS and higher viability when compared to suspension culture or culture on a mesh nanoinsert. Additionally after 14 days, nanofiber-encapsulated islets remained insulin positive and released insulin in response to glucose stimulation, while those cultured in suspension were weakly insulin positive and only observed at the periphery of the islets. A composite saccharide-peptide hydrogel was also developed whereby encapsulated rat islets remained viable up to 4 weeks, while the unencapsulated islets were mostly nonviable after 2 weeks.¹⁰⁷ Silk hydrogels have been studied for the encapsulation of mouse islets showing that the addition of collagen IV and laminin led to a twofold enhancement in insulin release compared with silk hydrogels alone.¹⁰⁸ In another study, murine ESC aggregates encapsulated in a collagen I gel exhibited enhanced expression of β -cell-specific genes and GSIS over 2D culture.¹⁰⁹ In this system, 46% of the clusters were positive for DTZ, a marker indicative of pancreatic β -cells, while only 7% were DTZ positive in 2D culture (however, expression levels were below that of freshly isolated islets). The GSIS levels were also higher by 3.3-fold in 3D compared with 2D cultures. Natural hydrogels, especially those

modified with ECM molecules, have been shown to enhance the viability and insulin secretion of encapsulated islets.

However, because natural polymers can be immunogenic and are not easily tuned (e.g., mechanical properties), synthetic polymers such as poly (ethylene glycol) (PEG) have been utilized to form hydrogels for pancreatic tissue engineering. Given the importance of cell–matrix interactions for islets and their precursors, synthetic hydrogels are often modified with ECM molecules like collagens I and IV or laminin, or small peptides. For example, insulin release from MIN6 cells encapsulated as single cells within PEG hydrogels was significantly enhanced with the addition of collagen IV, laminin, or combinations thereof.^{110,111} Interestingly, a 3:1 laminin:collagen ratio resulted in the highest insulin release and was sevenfold higher compared to cells in PEG-only gels, with no detectable insulin release from unencapsulated cells. Further analysis was performed to determine the key binding motifs in laminin (IKLLI, IKVAV, LRGDN, PDSGR, and YIGSR)^{112,113} and collagen (DGEA). Interestingly, only IKVAV showed enhancement in insulin secretion (three times more than unmodified) by encapsulated cells over 2 weeks. However, total insulin release from MIN6 cells in the gels with peptides mimicking laminin or collagen was two to five times lower than cells in gels with the entire proteins, suggesting that multiple domains of cell interaction and/or conformation of the domains are important. In a separate study, murine pancreatic precursor cells (isolated from day-15 dorsal pancreatic buds and dissociated into single cells) were encapsulated in PEG hydrogels for 1 week. These precursor cells were vimentin positive in 2D culture, but when encapsulated the β -cell-specific genes (e.g., *MafA* and *Ins*) were upregulated and the cells released insulin (but not in a glucose-responsive manner).¹¹⁴ The inclusion of collagen type I into the PEG hydrogels, however, led to glucose-responsive cells.¹¹⁵ Differentiation was further enhanced in 3D culture by inhibiting notch, which *in vivo* is responsible for maintaining undifferentiated cells, resulting in upregulation of pancreatic genes (*Hes1*, *Ins*, *PDX1*, *glut2*, *MafA*, and *GCG*) and higher GSIS values (Fig. 2e).¹¹⁶ Non-ECM-derived peptides have also been investigated as a means to enhance cell survival. For example, PEG hydrogels immobilized with antiapoptotic glucagon like peptide 1 and IKVAV improved the viability of encapsulated MIN6 single cells, but long-term survival remained low.¹¹⁷ Viability was also improved in MIN6 cells with the inclusion of a blocking peptide for interleukin-1 receptor and RGD when exposed to inflammatory cytokines.¹¹⁸ While PEG hydrogels have shown promise, a recent study has demonstrated that when formed by free radical polymerization, MIN6 cell viability is much lower in hydrogels formed from PEG diacrylates when compared to gels formed from a thiol-ene click chemistry,¹¹⁹ suggesting that the method to form hydrogels must be considered. Taken together, these studies reveal that synthetic hydrogels are promising platforms for pancreatic tissue engineering, but that the inclusion of ECM mimetics is critical to enhancing not only survival but also differentiation and function.

Another factor that has been considered in the death of cells in hydrogels is anoikis due to a lack of cell–cell contact or cell–cell signaling. Studies have shown that for MIN6 cells encapsulated in PEG hydrogels a higher concentration of cells at the time of encapsulation (2×10^7 cells/mL) leads to the formation of viable cell clusters while lower cell concentrations at the time of encapsulation (5×10^6 cells/mL) tend to result in cells remaining as single cells that eventually

die.¹²⁰ A higher concentration of cells at encapsulation also led to higher GSIS levels (e.g., 1.7 for 2×10^7 cells/mL and 0.8 for 6.7×10^6 cells/mL). Further, preaggregating MIN6 cells prior to encapsulation improved viability, with the cells remaining >95% viable after 3 weeks in culture.¹²¹ Similar results were observed with encapsulated mouse islets.¹¹⁰ These studies indicate that cell–cell communication, whether it be cell–cell contacts, paracrine signaling, or a combination, is important for cell viability and cell insulin secretion, especially for cells encapsulated in hydrogels.

A few studies have investigated coculturing islets with other cell types in hydrogels as a means to enhance islet function. For example, when cadaveric human islets were cultured in a collagen gel populated with fibroblasts, their GSIS levels were maintained after 1 week, but fell when cultured in a collagen matrix alone.¹²² Both conditions were nonetheless responsive to glucose, whereas 2D cultures were not. Similarly, mouse islets exhibited higher insulin release when cocultured with MSCs in a hydrogel containing collagen and laminin compared with those in a hydrogel containing just the ECM components.¹⁰⁸ Additionally, the insulin secretion from INS-1 cells cocultured with human umbilical vein endothelial cells (HU-VECs) was greater than when just INS-1 cells were cultured.¹²³ While the exact mechanisms remain to be elucidated, it is thought that basement membrane proteins secreted by endothelial cells, which have been shown to enhance the function of β -cells,⁶⁹ contribute to the increased insulin secretion observed in the coculture system *in vitro*. Additionally, MSCs are known secrete anti-inflammatory or antiapoptotic growth factors, which can enhance islet viability *in vitro*.

Preclinical Advancements

Several studies have investigated the ability of islets, which are precultured in various 3D environments *in vitro* and then transplanted, to reverse hyperglycemia in immune-compromised mice. For example, when murine islets cultured in PLGA scaffolds containing different ECM proteins were transplanted into mice with chemically induced diabetes, the condition was reversed as evidenced by lower blood glucose levels.¹²⁴ Of the ECM proteins tested, collagen IV led to the fastest response (basal blood glucose lowered from 300 mg/dL to below 200 mg/dL in ~ 4.4 days) compared with laminin and fibronectin, which required 27 days, and PLGA-alone scaffolds, which required 36 days, to lower blood glucose levels. In this same study, mice were subjected to a glucose challenge and islets on the collagen IV PLGA scaffolds reduced blood glucose levels within 60 min, while those on the fibronectin, laminin, and PLGA-alone scaffolds required ~ 120 min. Further, the presence of ECM improved neovascularization of the scaffolds, a critical factor for the long-term survival of transplanted islets. Another study using islets cultured on PGA scaffolds confirmed the importance of the 3D environment over injection of islets alone, which were precultured in 2D, showing a twofold reduction in blood glucose levels (257 mg/dL vs. 536 mg/dL) in nude mice.¹²⁵ Other studies have also shown that islets encapsulated in synthetic hydrogels are capable of reversing hyperglycemia in nude mice, while unencapsulated cells are not.^{121,126} For example, islets encapsulated in a saccharide-peptide hydrogel or in collagen-alginate microbeads and transplanted into nude mice reversed hyperglycemia (blood glucose <300 mg/dL)

for up to 4 weeks, while those grown in suspension culture prior to implantation could not.^{102,107} The time of the *in vitro* culture period prior to implantation has also been shown to be important. For example, human islets precultured in hydrogels for 2 weeks were able to reverse hyperglycemia in diabetic mice, but not after a 4-week preculture period.¹²⁷ In addition, transplanting PLLA/PLGA scaffolds with HUVECs and fibroblasts along with islets improved integration into the host vasculature and reduced blood glucose levels.⁹⁹ Implanted pancreatic precursor cells derived from hESCs without a scaffold have been shown to reverse hyperglycemia in nude mice, but take several weeks to have an effect.^{28,29} Combining these cells with a scaffold may be able to shorten the maturation time needed to achieve functional implants. Overall, these findings demonstrate the positive effect of 3D preculture and transplantation of islets in a 3D scaffold on the function of these cells *in vivo*. The 3D scaffold technology merits further investigation and extension to pancreatic stem or precursor populations as well as testing for long-term efficacy.

Concluding Remarks and Future Directions

Generally, islet transplantation has been successful for short periods of time. With recent improvements to the initial Edmonton protocol and the immunosuppressive cocktail, insulin independence has increased to 5 years post-transplantation in 50% of patients.^{7,128} However, long-term transplanted islets still lose viability and efficacy. The techniques described within this review could further improve the clinical outcome of islet transplantation by lengthening viability of islets *in vitro* prior to transplantation or *in vivo* post-transplantation and enhancing insulin secretion. In particular, a number of studies, both *in vitro* and *in vivo*, have demonstrated that islets, β -cells, and their precursors cultured in a 3D environment and with ECM that recapitulates the native islet exhibit improved viability and enhanced GSIS. These findings strongly point toward the importance of 3D contextual presentation of cell-matrix contacts. However, preclinical testing will be required to determine whether the significant improvements in functionality observed *in vitro* can extend to *in vivo* settings and for how long. Given the lack of available donor tissues and donor islets, it is imperative to extend these approaches to the directed differentiation of stem cells toward creating insulin-producing cells. Few studies point toward the importance of replicating the dynamics of the extracellular environment during organogenesis. Further work, however, is needed to identify suitable *in vitro* cues that drive stem cells into insulin-producing cells and the best method for presenting these cues within functional encapsulation materials. Ultimately, β -cells derived from ESCs or iPSCs will need to supplement and even surpass cadaveric islets as cell sources capable of reversing hyperglycemia in order to treat the number of patients suffering from type 1 diabetes. Incorporating the advancements in 3D culture techniques into directed differentiation protocols and transplantation procedures has the potential to make long-term β -cell replacement a reality. While not a focus of this review, the protection of insulin-producing cells from immune attack, either by microencapsulation of individual islets or macroencapsulation of multiple islets, will also need to be addressed to limit the need for immunosuppressive drugs.¹²⁹

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